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Thioredoxin reductase 1 deficiency enhances selenite toxicity in cancer cells via a thioredoxin-independent mechanism

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Synopsis

Selenium is an essential trace element in mammals, but is toxic at high levels. It is best known for its cancer prevention activity, but cancer cells are more sensitive to selenite toxicity than normal cells. Since selenite treatment leads to oxidative stress, and the thioredoxin system is a major antioxidative system, we examined the interplay between thioredoxin reductase 1 (TR1) and thioredoxin 1 (Trx1) deficiencies and selenite toxicity in DT cells, a malignant mouse cell line, and the corresponding parental NIH3T3 cells. TR1 deficient cells were far more sensitive to selenite toxicity than Trx1-deficient or control cells. In contrast, this effect was not seen in cells treated with hydrogen peroxide, suggesting that the increased sensitivity of TR1 deficiency to selenite was not due to oxidative stress caused by this compound. Further analyses revealed that only TR1-deficient cells manifested strongly enhanced production and secretion of glutathione, which was associated with increased sensitivity of the cells to selenite. The data uncover a new role of TR1 in cancer that is independent of Trx reduction and compensated for by the glutathione system. The data also suggest that the enhanced selenite toxicity of cancer cells and simultaneous inhibition of TR1 can provide a new avenue for cancer therapy.

Keywords

Selenium cytotoxicity; Thioredoxin reductase 1; Thioredoxin; Glutathione; Cancer; Selenoprotein

INTRODUCTION

Selenium plays an important role in decreasing the incidence of cancer and other human diseases [1–4]. More recently, epidemiological and genetic studies, as well as various molecular approaches and mouse models, have implicated selenium-containing proteins

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AUTHOR CONTRIBUTION

Ryuta Tobe, Min-Hyuk Yoo, Noelia Fradejas and Bradley A. Carlson performed the experiments. Soledad Calvo, Vadim N. Gladyshev, Min-Hyuk Yoo and Dolph L. Hatfield planned the experiments, analyzed the data and wrote the manuscript and the other authors, Ryuta Tobe, Noelia Fradejas and Bradley A. Carlson, also participated in planning the experiments, analyzing the data and writing the manuscript.

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(selenoproteins) in having roles in cancer prevention, including colon [5, 6], prostate [2, 7] and other cancer forms [8–10]. It should also be noted that small molecular weight selenocompounds, as well as selenoproteins, have been shown to play roles in cancer and other disease prevention and in-depth reviews have been written on this subject [11–13]. The fact that both these classes of selenium-containing components have anticarcinogenic properties has led to considerable debate in the selenium field regarding which forms of selenium (i.e., small molecular weight selenocompounds or selenoproteins) provide greater health benefits. In more recent years, selenoproteins have received by far more attention [14].

One selenoprotein that appears to participate in cancer prevention is thioredoxin reductase 1 (TR1) [15, 16]. TR1 is required for activation of the p53 tumor suppressor and for supporting other tumor suppressor activities [17], and is targeted by carcinogenic electrophilic compounds [18]. In addition, this selenoenzyme is one of the major redox regulators in mammalian cells and is expressed in all cell types, organs and tissues [15, 19, 20]. The targeted removal of TR1 in mice is embryonic lethal, and this protein also has roles in transcription, cell proliferation and DNA repair [21, 22]. The major function of TR1 in normal cells is to keep thioredoxin (Trx) in the reduced state; Trx then donates reducing equivalents to numerous cellular proteins, such as peroxiredoxins and methionine sulfoxide reductases [23]. In this manner, one of the major cellular redox systems, the Trx system, is regulated by TR1. The catalytic activity of TR1 that accounts for its function is governed by the selenocysteine (Sec) moiety, which is the C-terminal penultimate amino acid [24]. The above functions of TR1 provide strong evidence that this protein plays a highly significant role in maintaining cellular redox homeostasis and thus keeping cells in a healthy state. In addition, TR1 deficiency was reported to induce the cytoprotective transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2), which is well known to control gene expression involved with phase II detoxification and antioxidant enzymes against oxidative stress or chemical challenge [25, 26], but the role of Trx in the regulation of Nrf2 is not completely understood.

TR1 can be considered as having a split personality in that it has roles in preventing cancer and also in promoting and/or sustaining cancer [10]. Indeed, TR1 is over-expressed in many tumors and cancer cells [19]. This enzyme has been designated as a target for cancer therapy, since a variety of potent inhibitors of this selenoenzyme inhibited cancer progression [9–20]. Moreover, certain anticancer drugs targeting TR1 decreased its activity in malignant cells, and consequently, changed the cancer properties of such cells (see references 27–29 and references therein). In addition, TR1 has been shown to be uniquely over-expressed, as compared to other mammalian selenoproteins, in a variety of cancer cell lines [19]. Furthermore, its targeted removal using RNAi technology altered cancer-related properties of malignant cells demonstrating that tumorigenic and metastatic properties of some cancer cell lines are dependent on TR1 expression [30]. Down-regulation of TR1 in a mouse cancer cell line driven by oncogenic *k-ras* has revealed that TR1-deficient cells lose self-sufficiency of growth, have a defective progression in their S phase and exhibit a reduced expression of an enzyme involved in DNA replication, DNA polymerase- α [31]. These latter studies have shown that TR1 has major roles in several of the hallmarks of cancer described by Hanahan and Weinberg [32], including metastasis and self-sufficiency in growth signals [30].

Selenite can be used as a source of selenium for cell growth, but at higher levels this compound is toxic. Cancer cells are known to be more sensitive to selenite toxicity than normal cells, and the relative sensitivities of different cancers to this selenium anion also vary [33, 34]. Other reports have shown an inverse relationship between resistance to cytotoxic drugs and sensitivity to selenite in cancer cells [35, 36]. Selenite is thought to

induce apoptosis through oxidative stress [37, 38]. It has been proposed that the mechanism of selenite cytotoxicity involves the generation of reactive oxygen species (ROS) through intracellular redox cycling with oxygen and cellular thiols [39]. However, the reason why cancer cells are more sensitive to selenite than normal cells is not clear. TR1 is known to have a broad substrate specificity, and selenodiglutathione (GS-Se-SG) and selenite were reported to be substrates for mammalian TR1 in biochemical studies [40, 41] suggesting a role of TR1 in selenium metabolism or selenium cytotoxicity.

To elucidate the role of TR1 in selenite toxicity in cancer cells, we compared the relative selenite sensitivities of DT cells, a cancer cell line derived from NIH3T3 cells [42], to the parental cell line. DT and NIH3T3 cells were made deficient in TR1 or Trx1 expression, and the effects of selenite and hydrogen peroxide (H_2O_2) on the viability of these cells in relation to oxidative stress examined. These studies demonstrated that a TR1-deficient cancer cell line, designated DT/siTR1, was more sensitive to selenite than the corresponding Trx1-deficient or control cells and that the level of glutathione (GSH) in TR1-deficient cells was significantly increased compared to other cells. The data suggest that TR1 deficiency caused an increased production of GSH, which in turn caused an enhanced cytotoxic response to TR1 deficient cells.

EXPERIMENTAL

Materials

Products were purchased as follows: H_2O_2 , sodium selenite (Na_2SeO_3), reduced GSH, oxidized glutathione (GSSG), N-acetylcysteine (NAC), metaphosphoric acid, phthalaldehyde, N-ethylmaleimide (NEM), β -nicotinamide adenine dinucleotide phosphate reduced tetra sodium salt (NADPH), and guanidine hydrochloride from Sigma; BCA protein assay reagent and SuperSignal West from Thermo Fisher Scientific; PVDF membrane, NuPage 4–12% Bis-Tris gels, trypan blue exclusion test assay, Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic solution and fetal bovine serum from Invitrogen Life Technologies; primary antibodies for Trx1, glutathione reductase (GSR) and glutathione synthetase (GSS) from Abcam; primary antibody for glutathione s-transferase $\alpha 1$ (GST- $\alpha 1$) from Detroit R&D; and anti-rabbit HRP conjugated secondary antibody from Cell Signaling Technology. ^{75}Se was obtained from the Research Reactor Facility, University of Missouri, Columbia, MO. NIH3T3 cells and DT cells were obtained as previously reported [42].

Generation of TR1 and Trx1 deficient cells

Knockdown of TR1 in DT cells and preparation of the corresponding cell line encoding the pU6-m3 control vector that lacked the siRNA sequence were previously reported [31]. The control, stably transfected pU6-m3, in DT and NIH3T3 cells were produced as described [30]. Knockdown of TR1 and Trx1 in NIH3T3 cells and of Trx1 in DT cells were carried out as reported [30] with the following exceptions: Trx1 mRNA sequence, NM_011660, was surveyed for targeting its removal using the same strategy employed in generating TR1 knockdown DT cells [31]. The following nucleotide sequences were selected for targeting Trx1 mRNA: 5'-gctcagtcgttagaacat-3', 5'-gccactgcttaaggcaaa-3' and 5'-ccaactgccatctgattat-3'.

Culture of mammalian cells and cell viability

NIH3T3 and DT cells were grown at 37°C, 5% CO_2 in DMEM supplemented with 10% (v/v) fetal bovine serum and antibiotic-antimycotic solution that contains 10,000 units/ml of penicillin, 10,000 $\mu g/ml$ of streptomycin and 25 $\mu g/ml$ of amphotericin B. 5×10^5 Cells were incubated overnight and then treated with sodium selenite or H_2O_2 at the concentrations

given in the figures and/or figure legends for 24 h. NAC and GSH were added 30 min prior to sodium selenite treatment. Cell viability was measured by the trypan blue exclusion assay.

Quantification of GSH and GSSG

The concentrations of GSH and GSSG were measured as described previously using the fluorometric method [43]. Briefly, cells were washed with PBS and homogenized with phosphate-EDTA buffer (0.1 M sodium phosphate and 5 mM EDTA, pH 8.0). Protein was quantitated using BCA protein assay reagent and precipitated with 5% (w/v) metaphosphoric acid. Samples were mixed with 90% (v/v) phosphate-EDTA buffer and 100 µg o-phthalaldehyde (OPA) to measure GSH concentration. To measure GSSG, cell extracts were treated with 4 mM N-ethylmaleimide for 30 min at room temperature and mixed with 0.09 N NaOH and 100 µg OPA. Reaction mixtures were incubated for 15 min at room temperature. Fluorescence was measured at excitation and emission wave-length of 350 nm and 420 nm, respectively. Pure GSH and GSSG were used to generate standard curves.

Western blot analysis, ⁷⁵Se labeling and thin layer chromatography (TLC)

Techniques used for Western blot analysis have been described elsewhere [30, 31]. For ⁷⁵Se labeling, cells were seeded onto a 6 well plate (3×10⁵ cells/well) and labeled with ⁷⁵Se (40 µCi/well) (University of Missouri, Columbia, MO) for 24h. Media samples were collected, the cells washed with PBS and then harvested. Media and lysate samples were separated by TLC using silica gel 60 F254 plates in chloroform:methanol:H₂O (65:30:5). GSH and ⁷⁵Se were used as controls and authentic labeled selenodiglutathione was prepared by incubating ⁷⁵Se and GSH in buffer [9]. After running, the plate was stained with ninhydrin and the ⁷⁵Se-glutathione conjugate in media and cell lysate was identified by autoradiography [30]. ⁷⁵Se-Labeled proteins were electrophoresed and detected as described [30, 31].

RESULTS

Generation of TR1 and Trx1 deficient cells

We targeted the removal of TR1 or Trx1 in NIH3T3 and DT cells by examining three sites in both *TR1* and *Trx1* mRNAs and selecting those most effective for protein removal [30, 31]. NIH3T3 and DT cells were then stably transfected with the control construct, pU6-m3, designated NIH3T3/pU6-m3 and DT/pU6-m3, respectively, and with the most effective siTR1 and siTrx1 knockdown constructs, designated NIH3T3/siTR1, DT/siTR1 and NIH3T3/siTrx1 and DT/siTrx1, respectively. More than 90% of TR1 and Trx1 appeared to be removed in stably transfected cell lines as determined by Western blotting (Figure 1A).

Effect of selenite and H₂O₂ on TR1 deficient cells

Since DT cells were derived from NIH3T3 cells [42], the parental cell line provided a normal control for DT cells. We therefore compared these two cell lines in subsequent experiments. The relative sensitivities of both cell lines to selenite were examined (Supplementary Figure 1). Significant differences were observed at 5 and 10 µM sodium selenite, wherein the parental cell line was little affected by 5 µM selenite, but the malignant cell line showed a pronounced inhibitory effect. At 10 µM, both cell lines were highly sensitive, but DT cells showed higher sensitivity. As 5 µM selenite appeared to be fairly non-toxic to NIH3T3 cells, but an effective toxin to DT cells, this level of selenite was used in subsequent studies.

We next examined the sensitivities of NIH3T3, DT and TR1- and Trx1-deficient cells to selenite, using two methods, the trypan blue exclusion method and the MTT assay. Toxicity of sodium selenite was increased in a dose-dependent manner in both cell lines as was

similarly shown using both methods (Figure 1B and Supplementary Figure 2, respectively). Since significant differences between pU6-m3 control cells and TR1- and Trx1-deficient cells were observed at 5 μ M (Figure 1B), this level of selenite was used in subsequent studies. Viability of TR1- and Trx1-deficient parental cells, NIH3T3/siTR1 and NIH3T3/siTrx1, respectively, upon treatment with selenite was similar and about half that of the control parental cell line (Figure 1B and Supplementary Figure 2). The sensitivity of DT/siTR1 cells to selenite was greater than DT/siTrx1 and DT/pU6-m3 control cells, whereas Trx1 deficient cells were only slightly more sensitive than control cells (Figure 1B and Supplementary Figure 2). In addition, DT/siTR1 cells were proportionately more sensitive to selenite relative to their control cells than were NIH3T3/siTR1 cells relative to their control cells (compare Figure 1B and Supplementary Figure 2, upper panel to lower panel). The possible significance of these data is further considered below.

The finding that DT/siTR1 cells were far more sensitive to selenite than DT/siTrx1 cells was unexpected. This observation, the fact that TR1 is the primary reductant of Trx1 in normal mammalian cells [23], and the observation that selenite-treated malignant cells have been proposed to suffer from oxidative stress [44, 45], prompted us to determine whether the different sensitivities to selenite were due to cell death from oxidative stress or due to decreased growth rate. The three DT cell lines treated with buffer, selenite or H₂O₂ were examined (Figure 1C, upper, lower and middle panels, respectively). DT/siTR1 and DT/siTrx1 cells were dying when treated with selenite and the reduced viability observed in the presence of this compound was therefore due to cell death. On the other hand, cell morphologies of these three cell lines were similar in response to H₂O₂, suggesting that the reduced cell numbers observed in the presence of this oxidant were due to decreased growth rates.

Selenite treated TR1 and Trx1 deficient cells were further examined to assess whether the decreases in cell viabilities were caused by apoptosis (Supplementary Figure 3). The cell cycle was not affected by TR1 and Trx1 deficiency compared to control cells and the Sub-G1 population was not altered significantly by selenite treatment. These data suggest that the reduced viability of cells by selenite was not caused by apoptosis.

Effect of selenite and H₂O₂ on GSH metabolism

Since H₂O₂ did not appear to affect TR1- and Trx1-deficient cells, we examined whether selenite might affect the expression of enzymes involved in the GSH system, especially in TR1-deficient cells. Thus, mRNA levels of *Gss*, *Gsr* and *Gst- α 1* were examined in DT/siTR1 and DT/siTrx1 cells treated with selenite or H₂O₂ and compared to those in DT/pU6-m3 cells (Supplementary Figure 4). These enzymes are involved in the biosynthesis of GSH (GSS), reduction of GSSG (GSR), or conjugation of GSH to toxic metabolites (GST- α 1). Only the TR1-deficient cells exposed to selenite showed a dramatic increase in levels of all three mRNAs compared to treated and untreated control cells (Supplementary Figure 4). To assess protein expression, Western blot analysis was performed on these enzymes showing that the enhanced levels of mRNAs for these enzymes also reflected an increase in their protein expression (Figure 2A). Expression levels of GSS in DT/siTR1 cells were higher than in DT/pU6-m3 and DT/siTrx1 cells. Furthermore, TR1-deficient cells exposed to selenite showed an increase in levels of all three enzymes, especially in expression of GST- α 1 (compare Figure 2A, lane 5 to the other lanes). Trx1-deficient cells exposed to H₂O₂ showed higher levels of GSR and GSS than cells exposed to selenite (compare Figure 2A, lane 9 to lane 7 and 8, and Supplementary Figure 4A and 4B). Interestingly, the levels of enzymes involved in GSH metabolism in DT/siTR1 cells exposed to H₂O₂ were not as enriched as in cells exposed to selenite. These results suggest that TR1-deficient cells require higher GSH levels to compensate for the loss of TR1 than control and even Trx1-deficient cells, especially when they are exposed to selenite.

To further elucidate a possible protective role of GSH under conditions of selenite toxicity caused by TR1 deficiency, intracellular GSH and GSSG and extracellular GSH levels in DT/pU6-m3, DT/siTR1 and DT/siTrx1 cells grown in the absence or presence of selenite or H₂O₂ were examined by two different methods, the OPA histofluorescence method [43] and the tietze recycling method [46] (Figure 2B, 2C and Supplementary Figure 5, respectively). Similar levels of intracellular GSH and GSSG were observed by both methods following exposure to selenite and H₂O₂ with the exception that (and as anticipated) the amount of GSSG in selenite treated cells measured by the tietze method manifested much higher levels of GSSG (Supplementary Figure 5) when compared with the data obtained by OPA method (Figure 2B). This was due to the fact that the tietze method uses glutathione reductase (GSR) which is known to detect Se-GSH conjugates such as GSSeSG in addition to GSSG, and GSR metabolizes both GSSG and GSSeSG [47]. Intracellular levels of GSH in TR1 deficient cells were approximately three times higher than in control cells. Selenite treatment totally depleted GSH in all three cell lines, but these cells had somewhat similar levels of GSH upon exposure to H₂O₂ (Figure 2B, left panel). Intracellular levels of GSSG were low and similar in the three cell lines of control and selenite exposed cells, but showed dramatic increases in DT/siTR1 and DT/siTrx1 cells exposed to H₂O₂ compared to the corresponding control cells (Figure 2B, right panel); therefore, the high amount of GSSG in malignant cells was likely due to a compromised Trx system. Extracellular or secreted levels of GSH in DT/siTR1 cells were more than 3 times higher under normal growth conditions, and selenite treatment triggered the increased secretion of GSH in all three cell lines, especially in TR1 deficient cells (Figure 2C).

Effect of GSH on selenite toxicity

To verify the role of extracellular GSH in protection against selenite toxicity caused by TR1 deficiency, we pretreated cells with GSH or NAC which is known to affect the production of this antioxidant. All cell lines exposed to only GSH or NAC did not show observable differences in viability, but pretreatment of cells with GSH or NAC prior to selenite treatment caused massive cell death in all cell lines (Figure 3). The viability of all cell lines was less than 10% when compared with GSH or NAC treated cells not treated with selenite.

To examine the effect of extracellular GSH on selenite cytotoxicity, cells were treated with GSH and labeled with ⁷⁵Se (Figure 4). ⁷⁵Se-Conjugates were found in media and lysate samples by performing thin layer chromatography (Figure 4). The highest level of selenium glutathione conjugate (most likely selenodiglutathione (GS-Se-SG) (see Discussion)) was observed in TR1-deficient cells, and GSH treatment increased the selenium-glutathione conjugate levels, especially in the media of control and Trx-deficient cells (Figure 4A). DT/pU6-m3 control cells had an extremely low intracellular level of selenium-glutathione conjugate when compared with TR1 or Trx1 deficient cells, and GSH treatment significantly increased the intracellular level of selenium-glutathione conjugate in control cells but only slightly, if at all, in TR1 and Trx1 deficient cells (Figure 4B). As was previously reported, the addition of GSH resulted in increased levels of selenium-glutathione conjugate [40], but the increase did not appear to affect the expression level of selenoproteins (Supplementary Figure 6), whereas the addition of GSH or cysteine caused massive cell death in control, TR1 deficient and Trx1 deficient DT cells (Figure 3). These observations suggest that the increase in selenium-glutathione conjugate formation resulted in massive cell death in all the cell lines.

DISCUSSION

The Trx and GSH systems are regarded as major redox regulatory systems that maintain thiol redox homeostasis in mammalian cells. These systems are of particular importance to cancer cells since cancer cells are known to suffer from oxidative stress and rely on these

protective redox systems for survival [45]. In fact, enhanced Trx and GSH systems in tumor and cancer cells, their possible interrelationship and interdependence, and their roles as targets in cancer therapy have been recognized and examined in several studies [48–49]. We have been interested in the role of TR1 in malignant cells, as this selenoenzyme is enriched in many cancer cells and tumors, and appears to have opposing roles in both preventing and promoting/sustaining cancer (see Introduction).

We first observed that DT cancer cells lacking TR1 were far more sensitive to selenite exposure than the corresponding Trx1 deficient or control cell lines as well as the corresponding parental NIH3T3 cells. This surprising finding suggested that the role of TR1 is not limited to the control of the redox state of Trx1. The sensitivity of TR1-deficient cells to selenite relative to control cells was also far greater in DT cells than in NIH3T3 cells (Figure 1B). It should be noted that there is a discrepancy in the protective role of TR1 against selenite cytotoxicity. It was previously reported that selenium and selenodiglutathione were reduced by mammalian TR1 in the presence of NADPH [40, 41], implicating TR1 in selenium metabolism and/or cytotoxicity. Cancer cells were found to express increased levels of TR1, but many cancer cell lines are more sensitive to selenite toxicity than normal cells [33, 34], suggesting the involvement of another pathway that is affected in TR1 deficient cancer cells. TR1 is known to have a role in protecting cells from oxidative stress mainly via reduction of Trx1 [23, 50, 51]. In this regard, the differences in the consequences of TR1 and Trx1 deficiency upon selenite or H₂O₂ exposure were especially interesting and unexpected.

To examine whether oxidative stress was involved in selenium cytotoxicity, we exposed control and TR1 or Trx1 deficient cell lines to sodium selenite or H₂O₂, wherein the latter agent was used as a positive control of oxidative stress. Different responses to those stresses were observed. The reduced viability of DT/TR1 and DT/Trx1 deficient cells following treatment with selenite was due to cell death, but it was not caused by apoptosis (Supplementary Figure 3). In contrast, their diminished cell numbers following treatment with H₂O₂ were due to decreased growth rates, even though H₂O₂ is known to induce severe oxidative stress (Figure 1C). The levels of ROS in these three cell lines (i.e., DT/TR1 deficient, DT/Trx1 deficient and DT control cells) was assessed by DCFDA which is a procedure used for detecting general ROS. There were no significant differences in the cell lines up to 6 h following treatment with selenite, but ROS increased in DT/siTR1 cells at 24 h following treatment (Supplementary Figure 7). The mechanism of the increase in intracellular ROS in late stages of selenite-induced cell death in TR1 deficient cells requires further examination. H₂O₂ appeared to increase GSH levels in control and Trx1 deficient cells. As expected, this treatment caused an accumulation of GSSG in TR1 and Trx1 deficient cells (Figure 2B), demonstrating that the GSH system decreased oxidative stress allowing cells to survive, and this phenomenon even occurred in cells having a functional Trx system. There was no significant change in GSSG levels by selenite treatment again suggesting that these cells were not suffering from significant oxidative stress or at least that selenite was simply acting through generation of H₂O₂.

We further investigated the role of GSH in protecting against selenite toxicity in DT/siTR1 cells by examining proteins involved in GSH production, the effect of selenite treatment on intracellular and extracellular GSH levels and the effect of increased levels of extracellular GSH on selenite cytotoxicity. GSH levels in DT/siTR1 cells were about three times higher under normal growth conditions, but selenite treatment depleted intracellular GSH in all cells (Figure 2B). It was previously reported that deletion of TR1 in mouse hepatocytes induced the cytoprotective transcription factor, Nrf2, and accordingly increased expression of phase II detoxification genes such as GSTs [25], but the role of Trx1 in the induction of Nrf2 by TR1 deficiency was not examined in detail. We found that expression of GSS, GSR

and GST- α 1 was increased by selenite treatment (Figure 2A) and that the extracellular levels of GSH, especially in DT/siTR1 cells, increased whereas intracellular levels of GSH were not restored by an increase in these enzymes (Figure 2B, left panel, and 2C, right panel). These observations showed that selenite treatment induced the production and secretion of GSH in DT/siTR1 cells, but not in DT/siTrx1 cells, suggesting that the regulation of GSH production and secretion by TR1 deficiency was not via Trx1. Pretreatment of GSH or NAC, which is known to induce the production of GSH, caused massive cell death in all cell lines (control, TR1 and Trx1 deficient cells), whereas GSH and NAC itself did not affect cell viability. These latter observations suggested that extracellular levels of GSH had a direct relationship with selenite-induced cell death and an increase in production and secretion of GSH in TR1-deficient cells that caused a higher sensitivity to selenite treatment. It has been proposed that selenium uptake and accumulation are crucial to specific selenite cytotoxicity in cancer cells, and the addition of extracellular GSH or thiol compounds along with selenite increased selenite cytotoxicity suggesting selenite uptake is controlled by the extracellular environment [39]. Recently, it was shown that selenodiglutathione (GS-Se-SG) was efficiently taken up by the vacuolar ABC-transporter, Ycf1p, and that overexpression of Ycf1p resulted in much higher selenite toxicity in yeast [52]. TR1-deficient cells produced and secreted much more GSH, manifested a high level of selenodiglutathione in the media and a much higher induction of GSH production and secretion following selenite treatment than control and Trx1-deficient cells. Thus, the increased sensitivity of TR1-knockdown cells apparently was caused by increased GSH production and secretion.

Mammalian TR1 has been reported to directly reduce selenite to selenide and Se^0 species [41]. The latter selenium form may react with reduced glutathione to form GS-Se-SG that was proposed to be the major form of entry of selenocompounds into selenium metabolism [9, 53]. Furthermore, it was recently reported that other selenocompounds such as 2-butylselenazolidine-4(R)-carboxylic acid, 2-cyclohexylselenazolidine-4(R)-carboxylic acid and selenocystine were much more toxic to TR1-deficient cells [54], and BSO, which is known to inhibit GSH synthesis, has been shown to inhibit tumor growth in TR1 knockout mice and to cause cell death in TR1-deficient cultured cells [54, 55]. The involvement of Trx1, however, in these studies was not investigated.

In the absence of TR1 (or GSH as a second line of defense), a partially reduced selenium species could interact with reduced thiols in proteins whereby modifying critical cysteine residues and disrupting protein function. Therefore, further investigation of selenium metabolism in TR1- and Trx1-deficient cells following selenite exposure and the interplay of GSH and TR1 in selenium metabolism and selenium cytotoxicity may provide fruitful avenues for cancer therapeutic strategies [49, 50].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

GSH	Glutathione
GSSG	Oxidized glutathione
GSR	Glutathione reductase
GSS	Glutathione synthetase
GST	Glutathione S-transferase
NAC	N-Acetyl cysteine
Nrf2	Nuclear factor erythroid 2-related factor 2
OPA	o-phthalaldehyde
ROS	Reactive oxygen species
Trx1	Thioredoxin 1
TR1	Thioredoxin reductase 1

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Figure 1. Differential roles of TR1 and Trx1 in protecting cells from stress caused by selenite and H_2O_2 treatment

(A) Expression of TR1 and Trx1 in NIH3T3 and DT TR1 and Trx1 knockdown cells. NIH3T3 cells (left panel) and DT cells (right panel) were stably transfected with pU6-m3 (control), siTR1 or siTrx1 vectors and expression of TR1 and Trx1 levels analyzed by Western blotting as described in Materials and Methods. Actin was used as a loading control. (B) Cell viability was determined by counting of live cell using trypan blue. NIH3T3 and DT cells stably transfected with control vector, or with siTR1 or siTrx1 knockdown vectors, were treated with 5 μ M selenite for 24 h. Values show the viability ratio (%) compared with control cells and are the means $\pm$ S.D. of three independent experiments.

*Indicates a significant difference ($p < 0.05$). (C) DT control and TR1 and Trx1 deficient cells were treated with buffer (upper panel), 5 μM selenite (center panel) or 250 μM H_2O_2 for 24 h (lower panel), examined under the microscope and photographed. Bar (right, lowest panel) is 200 μm .

Figure 2. Effects of selenite and H₂O₂ on GSH metabolism

(A) DT/pU6-m3, DT/siTR1 and DT/siTrx1 cells were left untreated or treated with 5 μM selenite or 250 μM H₂O₂ for 24 h and protein expression levels of glutathione synthetase (GSS), glutathione reductase (GSR), and glutathione *S*-transferase-α1 (GST-α1) examined by Western blotting. Actin was used as a control. The numbers under each panel show the relative band intensities that were normalized to the levels of actin expression. (B) Intracellular levels of GSH and GSSG were measured by the fluorometric method and expressed as nmol per mg of protein; values are the means ± S.D. of three independent experiments. *Indicates significant difference ($p < 0.05$). (C) Cells were incubated for 0, 12

or 24 h and media samples collected at each time point to examine GSH secretion. Media samples from cells treated with 5 μ M selenite were prepared at 12 h to examine the effect of selenite on GSH secretion. The total GSH concentrations in media samples were analyzed fluorometrically. Values are the means $\pm$ S.D. of three independent experiments. *Indicates significant difference ($p<0.05$).

Figure 3. Effect of GSH and NAC on selenite-induced cell death

DT/pU6-m3, DT/siTR1 and DT/siTrx1 cells were initially exposed to 0.5 mM GSH, 0.5 mM NAC or buffer (designated B in the figure), and 30 min later were treated with 5 μ M selenite (designated Se in the figure). Viability was measured after 24 h of selenite treatment. Values show the viability ratio (%) compared with control and are the means $\pm$ S.D. of three independent experiments. *Indicates a significant difference ($p < 0.05$).

Figure 4. Formation of selenium-glutathione conjugate in TR1 deficient cells
 DT/pU6-m3, DT/siTR1 and DT/siTrx1 cells were treated with GSH and metabolically labeled with ⁷⁵Se for 24 hr, and media and cell extracts examined. (A) Extracellular and (B) intracellular selenite conjugated forms of GSH are shown which was observed by thin layer chromatography. In (C), authentic selenodiglutathione generated in the reaction containing ⁷⁵Se and GSH is shown wherein ⁷⁵Se (left panel) and ninhydrin stained GSH (right panel) were detected on the same thin layer chromatogram.